Identification of non-tuberculous mycobacteria: utility of the GenoType Mycobacterium CM/AS assay compared with HPLC and 16S rRNA gene sequencing

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Non-tuberculous mycobacteria (NTM) causing clinical disease have become increasingly common and more diverse. A new reverse line probe assay, GenoType Mycobacterium CM/AS (Hain Lifescience), was evaluated for identification of a broad range of NTM. It was compared with phenotypic (HPLC) and molecular (DNA probes, in-house real-time multiplex species-specific PCR, 16S rRNA gene PCR and sequencing) identification techniques, which together provided the reference ‘gold standard’. A total of 131 clinical isolates belonging to 31 Mycobacterium species and 19 controls, including 5 non-Mycobacterium species, was used. Concordant results between the GenoType Mycobacterium assay and the reference identification were obtained in 119/131 clinical isolates (90.8%). Identification of Mycobacterium abscessus and Mycobacterium lentiflavum by the assay was problematic. The GenoType Mycobacterium assay enables rapid identification of a broad range of potentially clinically significant Mycobacterium species, but some species require further testing to differentiate or confirm ambiguous results.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are increasingly important causes of clinical infections (Heifets, 2004). The increasing prevalence of disease due to NTM is related both to more intense interactions of humans with certain types of environment and to changes in population demographics, including a growing number of people who are immunosuppressed as a result of human immunodeficiency virus/AIDS, malignancy or medical intervention. Many recognized NTM are significant opportunistic pathogens in patients with severe immunosuppression, whilst others are emerging as causes of disease in immunocompetent individuals, including Mycobacterium ulcerans, Mycobacterium haemophilum and Mycobacterium malmoense (Brown-Elliott et al., 2002).

At least 60 NTM species are currently recognized as causative agents of human pathology, with variable severity and prognosis (Tortoli, 2003). Identification of isolates to the species level is important, as infections with different Mycobacterium species often require different management (Heifets, 2004) and pathogens must be distinguished from environmental NTM contaminants. Thus, timely and accurate identification of mycobacteria is required to guide therapy and for epidemiological purposes.

The GenoType Mycobacterium assay (Hain Lifescience) is a new commercial kit developed to identify NTM from cultures. It involves DNA amplification targeting the 23S rRNA gene region, followed by reverse hybridization to specific oligonucleotide probes immobilized on membrane strips. There are two kits – the CM (common mycobacteria) and AS (additional species) kits. The CM kit identifies 15 Mycobacterium species, including Mycobacterium tuberculosis complex, whilst the AS kit aims to discriminate 16 additional less common NTM pathogens. Previous evaluations have produced conflicting results, largely due to a relatively narrow selection of Mycobacterium species, different reference standards and the use of the first version of the assay (Ruiz et al., 2002; Padilla et al., 2004; Mäkinen et al., 2006; Russo et al., 2006). Therefore, this study aimed to compare the performance of the current GenoType Mycobacterium CM/AS assays with established NTM identification methods, including HPLC and 16S rRNA gene sequencing, using a broad range of newly described Mycobacterium species.

Abbreviations: ITS, intergenic transcribed spacer; NTM, non-tuberculous mycobacteria.
METHODOLOGIES

Isolates. Clinical NTM isolates referred to the New South Wales Mycobacterium Reference Laboratory in Sydney, Australia, were included in the study. A total of 131 clinical isolates and 19 control strains (14 Mycobacterium species and 5 other species) was used.

Mycobacterium species control strains were Mycobacterium chelonae ATCC 35752, Mycobacterium xenopi ATCC 19250, Mycobacterium fortuitum ATCC 6841, Mycobacterium simiae ATCC 25275, ‘Mycobacterium paraffinicum’ ATCC 12670, Mycobacterium scrofulaceum ATCC 19981, Mycobacterium nonchromogenicum ATCC 19530, Mycobacterium gordonae wild-type strain, Mycobacterium kansasii TMC 201, Mycobacterium avium ATCC 25291, Mycobacterium abscessus ATCC 19977, Mycobacterium intracellular ATCC 13950, Mycobacterium marinum wild-type strain and Mycobacterium asiaticum ATCC 25276. Negative controls were Corynebacterium pseudodiphtheriticum, Rhodococcus equi, Nocardia brasiliensis, Gordonia species and Tsukamurella species.

Mycobacterium species other than M. tuberculosis complex were selected from among isolates referred between January 2006 and February 2007, to provide the broadest available range of species. Isolates had been stored on Middlebrook 7H11 agar at 4°C.

Identification of NTM. All clinical isolates were subjected initially to HPLC, as described elsewhere (Butler et al., 1991; CDC, 1996). Additional methods were used, if required, to identify isolates not distinguished by HPLC, confirm unusual HPLC patterns or differentiate closely related NTM species.

AccuProbe gene probes (GenProbe), which target the 16S rRNA gene without amplification, were used to confirm HPLC profiles and phenotypic characteristics consistent with M. avium, M. intracellulare, M. gordonae and M. kansasii (species for which AccuProbe probes are available). An in-house real-time multiplex PCR assay, using primers and species-specific TaqMan probes targeting the intergenic transcribed spacer (ITS) region of the 16S–23S rRNA gene, was adapted from the method of Xiong et al. (2006) for use with a SmartCycler II system (Cepheid). This assay was used to determine the species of NTM isolates with growth characteristics and HPLC profiles consistent with M. avium/M. intracellulare or M. abscessus/M. chelonae. Isolates that could not be identified with these methods were subjected to 16S rRNA gene PCR and sequencing (Relman, 1993).

Genotype Mycobacterium CM and AS assays. Isolates on solid medium were prepared by suspending a loopful of the bacteria in 1 ml distilled water. DNA extraction was performed by sonication for 15 min, followed by heating to 100°C for 15 min. Samples were then centrifuged at 13 400 for 2 min and the supernatant was used for the assay.

The GenoType Mycobacterium CM/AS assay was performed according to the manufacturer’s instructions. The process involved PCR amplification, hybridization of PCR products to probes bound to test strips and detection of bound products.

The test strips were fixed on a data sheet. Development of conjugate, universal and genus control lines were assessed carefully for each isolate. Reactions with probe lines were recorded. In accordance with the manufacturer’s instructions, only bands whose intensities were as strong as or stronger than the universal control line were considered.

Any isolate not identified by the GenoType CM assay was tested with the GenoType AS assay. Assays were performed and interpreted without knowledge of the previous identification.

Discrepant results. Discrepancies between the results of HPLC, AccuProbe, real-time PCR and the GenoType Mycobacterium assay were resolved by 16S rRNA gene PCR and sequencing. Sequences were aligned with known sequences from the RIDOM (ribosomal differentiation of medical microorganisms, http://www.ridom-rdna.de/) and GenBank databases, using Biomanager (Australian National Genomic Information Service, http://www.angis.org.au/). Sequence similarity of >99% was expected for species identification. Isolates identified as M. abscessus/M. chelonae, which have undistinguishable 16S rRNA gene sequences, were differentiated using the real-time species-specific PCR described above. Identification of other species with identical 16S rRNA gene sequences was resolved with PCR and sequencing of the 16S–23S ITS region (Roth et al., 1998). The final sequencing result(s) was taken as the definitive identification.

RESULTS AND DISCUSSION

All control strains of mycobacteria were correctly identified with the GenoType Mycobacterium assay except for M. xenopi ATCC 19250, which was identified only to genus level, although the assay included relevant species-specific probes. All negative-control strains reacted with the universal control probe only, indicating the presence of a Gram-positive bacterium with high mol% G+C content, not a Mycobacterium species. All clinical isolates reacted with the universal and genus control probes, confirming all isolates as belonging to the genus Mycobacterium.

The GenoType Mycobacterium CM strip identified 87/131 clinical isolates (66.4%) correctly to species level. The AS strip was used to identify the remaining 44 isolates, including 9 for which the CM strip could not distinguish between 2 closely related species, namely M. marinum/M. ulcerans (7 isolates) and M. haemophilum/M. malmoense (2 isolates). In total, the GenoType assay correctly identified 119/131 clinical isolates (90.8%: Table 1), 99 to species level and 14 to genus level only, whilst 6 were identified to 1 of 2 or 3 species (i.e. 2 Mycobacterium genavense/Mycobacterium triplex and 4 Mycobacterium peregrinum/Mycobacterium alvei/Mycobacterium septicum).

The AS strip assay failed to identify six isolates for which there were species-specific probes: a single isolate of M. genavense and one of two Mycobacterium hecshornense, which did not react with any species-specific probes; one M. simiae, and three of five Mycobacterium lentiflavum isolates, which bound weakly to the probe at position 6 on the strip, which is required for identification of this species. If weak binding at this probe site was accepted as a positive reaction, all three M. lentiflavum isolates would have been correctly identified. Sequence variation in the probe regions (23S rRNA gene) may explain the failure to hybridize with corresponding species-specific probes. The 16S rRNA gene sequences obtained from these isolates showed 99–100% identity with corresponding species in the RIDOM database.

Six isolates were misidentified by the GenoType assay, including three M. abscessus isolates identified as M. chelonae, based on the only reaction that differentiates them, namely binding (of M. abscessus DNA) to the probe at position 6 on the membrane strip. Weaker binding of
the PCR product at this probe line, compared with the universal control, was interpreted as negative, in accordance with the manufacturer’s instructions. The interpretation of the assay for differentiation of *M. abscessus* and *M. chelonae* and identification of *M. lentiflavum* may need to be reviewed so that any detectable binding at probe 6 is interpreted as a positive reaction. Two isolates of *Mycobacterium sphagni* (not included in the GenoType assay) were misidentified as *Mycobacterium mucogenicum* and one of *Mycobacterium szulgai* (for which relevant probes are included) as *M. scrofulaceum*.

Other studies that have compared this assay with a variety of phenotypic and genotypic methods have generally reported better results than our findings. Evaluations of an earlier version, which identified only 13 *Mycobacterium* species (Mäkinen et al., 2002; Padilla et al., 2004; Ruiz et al., 2002; Sarkola et al., 2004), showed that the assay correctly

<table>
<thead>
<tr>
<th>Species</th>
<th>Total no. of isolates</th>
<th>No. correct by GenoType*</th>
<th>GenoType for incorrectly identified isolates</th>
<th>No. correct by HPLC*</th>
<th>Supplementary identification methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DNA probe (correct/ performed)†</td>
<td>Species-specific PCR (correct/ performed)‡</td>
<td>16S rRNA gene sequencing performed</td>
</tr>
<tr>
<td><em>M. abscessus</em></td>
<td>8</td>
<td>5</td>
<td><em>M. chelonae</em></td>
<td>7</td>
<td>–</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>10/12</td>
<td>6/6</td>
</tr>
<tr>
<td><em>M. avium</em>†</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>1/1</td>
</tr>
<tr>
<td><em>M. paratuberculosis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. celatum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. chelonae</em></td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>10/10</td>
</tr>
<tr>
<td><em>M. flavescens</em></td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td><em>M. gastri</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. genavense</em></td>
<td>1</td>
<td>0</td>
<td><em>Mycobacterium</em> spp.</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>6/7</td>
<td>–</td>
</tr>
<tr>
<td><em>M. haemophilum</em></td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. heckeshornense</em></td>
<td>2</td>
<td>1</td>
<td><em>Mycobacterium</em> spp.</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><em>M. interjectum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. intracellular</em></td>
<td>17</td>
<td>17</td>
<td>15</td>
<td>4/9</td>
<td>5/7</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2/2</td>
<td>–</td>
</tr>
<tr>
<td><em>M. lentiflavum</em></td>
<td>5</td>
<td>2</td>
<td><em>Mycobacterium</em> spp.</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. mucogenicum</em></td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td><em>M. nebraskense</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. neoaurum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. nonchromogenicum</em></td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>–</td>
<td>–</td>
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<tr>
<td>‘M. paraffinicum’</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. peregrinum/M. septicum</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em></td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. simiae</em></td>
<td>1</td>
<td>0</td>
<td><em>Mycobacterium</em> spp.</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td><em>M. sphagni</em></td>
<td>2</td>
<td>0</td>
<td><em>M. mucogenicum</em></td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td><em>M. szulgai</em></td>
<td>1</td>
<td>0</td>
<td><em>M. scrofulaceum</em></td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td><em>M. terrae</em></td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. triplex</em></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. ulcerans</em></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total no. (%)</td>
<td>131 (100)</td>
<td>119 (91)</td>
<td>92 (70)</td>
<td>22/30</td>
<td>30/32</td>
</tr>
</tbody>
</table>

*All isolates were tested with the GenoType Mycobacterium assay and HPLC. Identifications for the GenoType assay were correct if isolates were identified to the species level where there were species-specific probes or to the genus level in the absence of species-specific probes.

†Accuprobe (GenProbe) targets *M. avium, M. intracellulare, M. kansasii* and *M. gordonae*. Results shown are for species within the identification range of the test.

‡In-house real-time multiplex PCR targeting the 16S–23S rRNA gene ITS region, with primers and probes for *M. avium, M. intracellulare, M. abscessus* and *M. chelonae*. Results shown are for species within the identification range of the test.
identified 89.3–100 % of isolates. The current assay, with the AS strip, identifies 31 species. In a prospective evaluation, 96 % of isolates were correctly identified (Mäkinen et al., 2006) when compared with 16S rRNA gene sequencing. The current assay was also compared with biochemical methods, HPLC, INNO-LiPA MYCOBACTERIA (Innogenetics NV) and 16S rRNA gene sequencing (Russo et al., 2006). The sensitivity and specificity of the CM strip were 97 and 92.4 % and of the AS strip were 99.3 and 99.4 %, respectively, compared with reference methods.

Differences in isolate selection may partly explain the variation in assay performance seen in previous studies. Padilla et al. (2004) found that the diversity of isolates tested influenced the results of their evaluation. They noted that, when the subgroup of isolates in the identification range of the assay was evaluated separately, the accuracy of the GenoType assay increased from 91 to 96.8 %. In contrast to previous evaluations, our study included many *Mycobacterium* species that are infrequently encountered in clinical specimens, rather than consecutive isolates. A total of 8 of the 31 species represented among our clinical isolates were not identifiable by the GenoType assay.

The wide range of *Mycobacterium* species selected for our study was also reflected by a lower proportion (66 %) of isolates identified with the CM strip alone, compared with up to 88 % of clinical isolates reported in other studies (Mäkinen et al., 2006). Twenty per cent of AS strips used on our isolates were to differentiate pairs of species that were not distinguished by the CM kit. However, some of these pairs can be differentiated by their phenotypic characteristics without the need for the GenoType AS assay. For example, *M. marinum* and *M. ulcerans* share the same 23S rRNA (and 16S rRNA) gene sequences but can be differentiated by growth conditions and pigment production.

Geographic sequence polymorphisms in mycobacterial species may also affect assay performance (Legrand et al., 2000). Most published evaluations have been carried out in Europe (Mäkinen et al., 2002, 2006; Ruiz et al., 2002; Padilla et al., 2004; Sarkola et al., 2004; Russo et al., 2006). As far as we are aware, there has been limited evaluation of the diagnostic performance of the GenoType assay on *Mycobacterium* species originating elsewhere.

Differences in reference ‘gold standards’ used in previous studies could contribute to reported differences in the diagnostic performance of the assay. Molecular identification tools have revolutionized bacterial taxonomy and identification methods. However, analysis of 16S rRNA gene sequences has limitations because of the low level of polymorphism between some closely related species. No single target gene can accurately distinguish all *Mycobacterium* species, and Devulder et al. (2005) have suggested that a combination of genes (such as 16S rRNA, hsp65, rpoB and sod) be used. However, more sequence data for these genes, in publicly available databases, is needed to improve their utility for species identification. Our study relied on the most commonly used targets – the 16S rRNA gene and the ITS region of the 16S–23S rRNA gene. The latter contains sufficient interspecific polymorphism and intraspecific conservation to make it an appropriate target for identification of most *Mycobacterium* species (Roth et al., 1998). Nevertheless, the use of multiple targets could increase the accuracy of species identification, particularly when a relatively wide range of species is under investigation, as in our study.

The utility of laboratory methods used to identify NTM in this study is compared in Table 2. The GenoType Mycobacterium assay has a narrower identification range than HPLC but is less technically demanding. Compared with other molecular assays, with the exception of PCR and sequencing, the GenoType assay has a broader identification range. The advantages of this assay over PCR and sequencing are its shorter turnaround time and the lower level of technical expertise required. The GenoType Mycobacterium assay requires a specialized temperature-regulated shaker, which increases the initial outlay. However, the equipment cost is considerably less than that

### Table 2. Laboratory methods for identification of mycobacteria

<table>
<thead>
<tr>
<th>Assay</th>
<th>Target</th>
<th>No. of species identified</th>
<th>Turnaround time</th>
<th>Cost</th>
<th>Technical expertise</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenoType Mycobacterium</td>
<td>23S rRNA gene</td>
<td>31</td>
<td>5 h</td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>HPLC</td>
<td>Cell-wall mycolic acid</td>
<td>Numerous*</td>
<td>5 h</td>
<td>Low†</td>
<td>High</td>
</tr>
<tr>
<td>AccuProbe</td>
<td>16S rRNA gene</td>
<td>5‡</td>
<td>2 h</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Real-time species-specific PCR</td>
<td>16S–23S ITS</td>
<td>5§</td>
<td>2 h</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>PCR and sequencing</td>
<td>16S rRNA gene, 16S–23S ITS</td>
<td>Numerous‖</td>
<td>3 days</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

*Limited by the library of HPLC profiles.
†Excluding the initial cost of equipment.
‡Specific probes for *M. avium, M. intracellulare, M. kansasii, M. gordonae* and *M. tuberculosis* complex.
§Limited by the number of species-specific primers and probes. This study used primers and probes for *M. avium, M. intracellulare, M. abscessus* and *M. chelonae*.
‖Limited by the number and quality of sequences submitted to sequence databases.
required for sequencing or HPLC, although both of the latter have many other applications.

The main limitation of this study was its retrospective nature. Stored isolates were used and assays were performed from cultures on solid medium only. Other studies have evaluated the GenoType Mycobacterium assay on NTM cultures from liquid media and showed 91–100 % concordance with conventional identification methods (Ruiz et al., 2002; Padilla et al., 2004). This study was also conducted on a number of relatively rare or newly identified NTM species rather than on consecutive isolates and thus probably underestimates the performance of the assay for routine diagnostic use. We did not evaluate the ability of this test to detect mixed cultures.

In conclusion, the GenoType Mycobacterium assay applied to clinical NTM isolates demonstrated rapid and accurate identification of a broad range of NTM compared with phenotypic and other molecular diagnostic techniques. This assay is relatively easy to use and does not require expensive equipment. However, identification of certain species, such as M. lentiflavum and M. abscessus, may be problematic. In addition, supplementary tests are needed for some isolates to resolve ambiguous identifications or for Mycobacterium species outside the identification range of the assay.

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REFERENCES


